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Assessing population substructure of whitebark pine with uniparentally inherited markers

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" Geographic Patterns of Genetic and Adaptive Variation in Northern Rocky Mountain WhitebarkPine"
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Final Report

Title: Assessing Population Substructure of Whitebark Pine with Uniparentally Inherited Markers

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Several allozyme studies have studied whitebark pine genetic structure at various spatial scales: regional diversity (Yandell 1992, Jorgensen and Hamrick 1997), local populations (Bruederle et al. 1998), and stand structure (Furnier et al. 1987, Rogers et al. 1999). Bruederle et al. (1998) sampled nine populations of whitebark pine in the Yellowstone area. They found little genetic substructuring among populations relative to other pines. Most genetic variation occurred among individuals within a population. Reports on range-wide and regional structuring varied. Yandell (1992) reported a relatively high differentiation (F_{st} =0.191) among isolated Great Basin and Sierran populations. Jorgensen and Hamrick (1997) found little genetic substructuring (G_{st}=0.034) throughout the range. The large differences in these F_{st} values may be attributable to the differences in study areas. The Yandell (1992) study included Sierran whitebark pine and isolated populations across the Great Basin, whereas Jorgensen and Hamrick (1997) sampled in more continuous stands across most of whitebark pine's range. At the local scale, Furnier (1987) found most genetic variation among clumps (individual trees that have growth together). Individuals within clumps were often related. This could result from nutcracker seed collection and caching behavior. Rogers et al. (1999) found similar results among both tree-form clumps and krummholz form. Most genetic differentiation was found among clumps (F_{st} = 0.334) with mainly full- and half-sibling composition in clumps. Differentiation among watersheds was small (F_{st} = 0.004).

Cost and speed make allozyme assays ideal for many genetic analyses. However, the low heterozygosity found in allozymes limits their use in intraspecific population genetics (Cervera et al. 1997). For whitebark pine, another disadvantage is that allozymes cannot distinguish between the two mechanisms of gene flow: seed dispersal via Clark's

nutcracker and wind-dispersed pollen. Recently, molecular genetic techniques have been developed to address questions about these processes. Uniparental inheritance of chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) among the Pinaceae provides a unique opportunity to distinguish between the two mechanisms of gene flow, pollination and seed dispersal (Wagner 1992).

Molecular Approach

Chloroplast Markers

Compared to allozyme data, highly polymorphic microsatellite loci may offer higher resolution for detection of genetic structure in whitebark pine. They consist of repeat motifs of one to five nucleotides. Microsatellites are believed to be neutral loci that have relatively little effect on fitness (Kashi et al. 1997). The recent development of chloroplast (cpDNA) and nuclear microsatellite markers in conifers (Powell et al. 1995, Vendramin et al. 1996, Echt et al. 1999) has provided a powerful tool for population genetic studies. Primers developed by Vendramin et al. (1996) have been used for various conifer species. Paternity analysis was studied in *Abies alba* (silver fir) between two isolated, old-growth individuals 150 meters apart. Genotypes from two microsatellite loci revealed most of paternal contributions in the regeneration came from the two old-growth trees, suggesting a high level of inbreeding. (Ziegenhagen et al. 1998). Another study used chloroplast microsatellites to reveal genetic diversity in *Pinus pinaster* (maritime pine). Two sites had the greatest diversity. These stands are apparently refugia from the last ice age; thus they are important to genetic conservation (Vendramin et al 1998).

Mitochondrial Markers

Seed dispersal and genetic structure of whitebark pine are influenced by Clark's nutcracker caching behavior (Tomback 1998). Seeds (ca. 35-150) are extracted from cones, stored in the sublingual pouch, and transported up to 12 km by the Clark's nutcracker. A 1 to 3 cm excavation is made in the soil where it stores 1-15 seeds (Vander Wall and Balda 1981, Tomback 1998). Seeds not retrieved before the following year may germinate. Although these studies and allozyme work has provided a understanding of how Clark's nutcracker contributes to the family structure within individual stands, neither can be extrapolated to determine how the bird's have influence whitebark pine population gene flow and colonization With maternally inherited mitochondria, mtDNA is passed on only via seed (Wagner 1992). This is direct link to nutcracker dispersal. Such a marker would be an informative tool in understanding the combined effect of Clark's nutcracker-assisted seed dispersal, caching behavior, and stand establishment.

In pines, gene flow via wind and bird-dispersed seeds has shown to have less dispersal capability than pollen (Dong and Wagner 1993, Latta and Mitton 1997, Latta et al. 1998). Consequently, more genetic differentiation should be apparent using mtDNA markers associated with seed. Latta et al (1998), measured introgression of two races of ponderosa pine (*Pinus ponderosa*) using mtDNA and cpDNA markers. Trees were sampled across a zone of hybridization in western Montana. Based on frequency of organellar haplotypes, it was concluded that most of the introgression resulted from pollination via prevailing westerly winds. Seed movement apparently had little effect on gene flow, resulting in a steep cline in the zone of introgression (Latta et al. 1998).

Several studies have used mtDNA to calculate population structure of conifer species (Dong and Wagner 1993, Latta and Mitton 1997, Latta et al. 1998, Wu et al. 1998). For example, Latta and Mitton (1997) used *Nad*1 gene introns (Demesure 1995) in limber pine (*Pinus flexilis*) population genetics. A comparison of allozyme, RAPD, cpDNA, and mtDNA markers were used to assess population genetics. Allozyme techniques showed the least differentiation among populations, and mtDNA haplotypes showed the greatest. Mitochondrial haplotypes were much more differentiated (F_{st} = 0.679) than cpDNA (F_{st} = 0.013). The results indicated that pollen is the major source of gene flow in limber pine.

Whether whitebark pine seed and pollen movement is similar to limber pine remains to be determined; however, both limber and whitebark pine have similar life histories, and Clark's nutcracker has a major importance in seed dispersal in limber pine as well (Tomback and Linhart 1990). A notable difference between these two species occurs in their range. Whitebark pine is specialized to exist in subalpine habitats lends itself to a relatively patching range, whereas limber pine has a more widespread range with fewer expected barriers to gene flow.

Methods and Avenues for Research

The first objective to this project was to screen primers for variation in cpDNA and mtDNA. Twenty microsatellite primers developed for *Pinus thumbergii* were tested on whitebark pine. Our research has identified three microsatellite chloroplast markers out of 20 that produce polymorphic bands in whitebark pine. Preliminary findings show that individuals from Idaho and the Oregon Cascades have substantial variation within populations. In the mitochondrion, two variable sites have been identified through

sequencing gene spacers (introns) from primers developed by Wu et al. (1998) for California closed cone pines: knobcone (*Pinus attenuata*), Monterey (*P. radiata*), and bishop (*P. muricata*). The current focus is gaining an idea of how the alleles in both mtDNA and cpDNA are distributed in the Northern Rocky Mountains and the Northern Cascades. When the distribution and spatial scale is determined for these loci, an experimental design can be setup for the most informative results.

Population substructure (F_{st}) and Gene flow (N_m) can be estimated with mtDNA and cpDNA markers. The equation developed by Ennos (1994) models how F_{st} of organellar DNA can be used to estimate F_{st} of nuclear (biparental) DNA, where $F_{st(b)}$ is the biparental (nuclear) estimate, $F_{st(p)}$ pollen, and $F_{st(m)}$ mitochondrial.

$$(1/ F_{st(b)}-1) = (1/ F_{st(p)}-1) + (1/ F_{st(m)}-1)$$

Hypotheses being tested

Analyses with mitochondrial markers and chloroplast microsatellites can help answer several key questions about population genetics in whitebark pine. Based on similar techniques employed in studies of other conifers and the knowledge gained from the previous allozyme studies, four hypotheses can be postulated.

1. *Due to differences in mutation rates among mtDNA and cpDNA genomes and the properties of cpDNA microsatellites, more variable loci and greater numbers allele per locus in cpDNA are expected.* Microsatellite DNA have one of the highest mutation rates due to slippage of polymerase during replication. Plant mtDNA has a slow mutation rate, 5 to 10 times slower than mtDNA in animals (Ennos 1999).

2. *As allozyme data have shown, genetic variation should exist within populations. However, we expect to find large-scale substructuring among isolated and geographically distant populations.* It is intuitive to think that with whitebark pine's large range and patch distribution that there are some geographic barriers to gene flow.
3. *Greater dispersal distances and gene flow are predicted for pollen (cpDNA). Seed dispersal via the Clark's nutcracker should have shorter dispersal distances and more substructuring.* This hypothesis is base on cpDNA and mtDNA analyses in ponderosa pine and limber pine (Latta 1998, Latta and Mitton 1997).
4. *Alternatively, elevational differences may influence phenology of flowering, which may create barriers in pollination of neighboring populations.* If there are such barriers we should see cpDNA differentiation at differing elevations. This can be tested by incorporating neighboring populations existing at different elevations.

Significance

Maintaining genetic diversity is critical for species to evolve under changing environmental conditions (Hamrick 1996). Currently, whitebark pine is undergoing strong selection pressure for resistance to blister rust. Planning is underway to begin a breeding program that identifies resistance individuals through artificial inoculation with blister rust (*Cronartium ribicola*).

An understanding of whitebark pine genetic structure can provide a strong biological foundation for breeding programs in blister rust resistance. A successful breeding program uses genetically diverse breed lines so resistant stock is closely match

with environment. Our research can provide insight into where to gather whitebark pine seed for breeding, and which trees should be bred to maintain the existing genetic structure.

Along with adaptive trait research that is currently underway, we think this research will be informative in refining seed zone guidelines and management units for whitebark pine. The data could also alert us to populations which may be undergoing inbreeding depression (e.g. low genetic variation) and unique divergent population that may warrant special attention.

Results

cpDNA

- DNA extraction has been optimized for whitebark pine needle tissue.
- Development and tested microsatellite analysis with PE ABI 377 DNA sequencer.

Chloroplast DNA microsatellite primers tested:

Primer	# of alleles found		Primer	#of alleles found
Pt1254	No PCR product		Pt71873	1
Pt9383	1		Pt63718	1
Pt15169 *	6		Pt71936	2
Pt26081	1		Pt79951	1
Pt30204 *	5		Pt87268	1
Pt36480	1		Pt100783	1
Pt41093	No PCR product		Pt102584	No PCR product
Pt45002	No PCR product		Pt107148	1
Pt48210	1		Pt107517	1
Pt51873	1		Pt109567	1
Pt71873	1		Pt110048	1

*sequenced to test for homoplasy

mtDNA

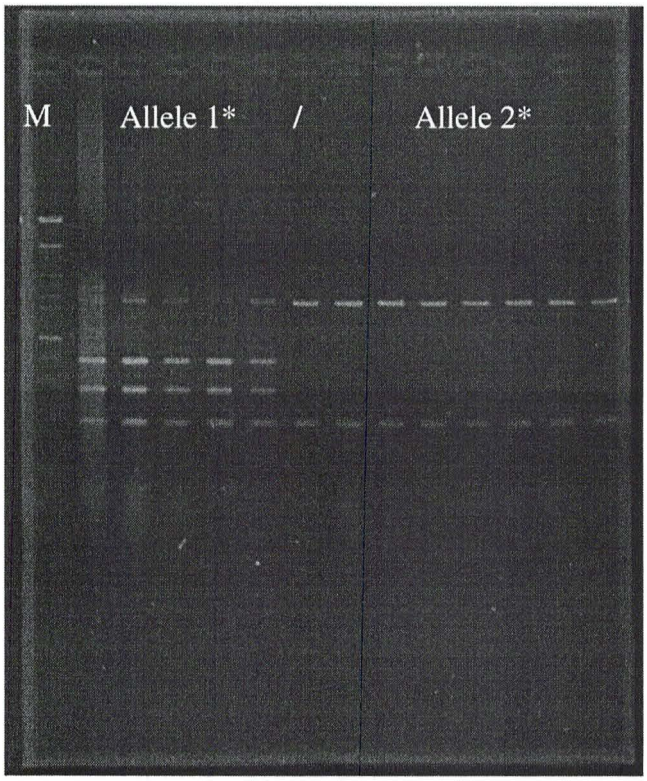
Mitochondrial DNA gene introns sequenced:

Gene intron (*Polymorphic)	Sequence length (base pairs)
Atp 6	~600
Cox I	~1450
Cox 3	~700
Nad 4a	~800
Nad 5a *	~1000
Nad 5d *	~1000

- Nad 4a primers resulted in poor PCR amplification. New primers are in the process of being developed from whitebark pine DNA sequence data.
- Nad 5a was found to be polymorphic; this polymorphism corresponds with a restriction site, which has allowed us to survey over 200 trees from the Cascades, Sierras and Rocky Mountains. (See Fig. 1 below)

- Nad 5d was also found to be polymorphic, but no available restriction enzyme recognizes the two different base pair substitutions. In order to survey the distribution of this mutation, two dye-label primers (fam and hex) were designed to pair with the polymorphic nucleotides found in sequencing. Thus over 200 trees have also been surveyed for this genetic difference.

Figure 1. RFLP of Nad 5a intron cut with Mse I restriction enzyme.



*Allele 1 distribution: Sierras and Southern Cascades to Mount Rainier. Allele 2: Rocky Mtns. and Northern Cacades

Landscape analysis

The two mitochondrial markers we have developed allow a range-wide interpretation of the genetic architecture of whitebark pine. Allele 1 of the Nad5a intron

has a distribution exclusive to the Northern Rocky Mountains and the Northern Cascades. Allele 2 is distributed from Mount Rainier south into the California Sierras (Fig. 1). A zone of introgression of these alleles exists between Mount Rainier and Lake Chelan. The narrowness of the contact zone between the two Nad5a alleles supports earlier observations that Clark's nutcrackers do not disperse seeds more than approximately 20 kilometers.

The Nad5d mitochondrial locus appears to be polymorphic throughout its range, which will provide the allelic diversity needed to calculate maternal gene flow. This latter work is supported by other funding sources. Allelic distribution of cpDNA markers seems to be more homogeneous, suggesting pollen dispersal is a major role in gene flow.

Continued Research

The introgression zone of mitochondrial alleles we have discovered provides information on not only population structure and gene flow, but also Clark's nutcracker behavior and flight patterns. Currently, additional sampling is underway within this introgression zone. In a continuation of this work, funded from other sources, both cpDNA and mtDNA markers are being used to evaluate how wind-dispersed pollen and bird-dispersed seed influence population genetic structure and gene flow at a local scale. The results described above will be the basis of a refereed publication on mitochondrial genetic patterns in whitebark pine. USDA funding (RJVA- INT- 95123) will be gratefully acknowledged.

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